The defensive role of nonspecific lipid-transfer proteins in plants

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Plant nonspecific lipid-transfer proteins stimulate the transfer of a broad range of lipids between membranes *in vitro*. In view of their ability to inhibit bacterial and fungal pathogens, their distribution at high concentrations over exposed surfaces and in the vascular system, and the response of *Ltp*-gene expression to infection with pathogens, they are now thought to be active plant-defense proteins.

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A range of plant-defense proteins is toxic or inhibitory towards other organisms, including bacterial and fungal pathogens. For example, the so-called nonspecific lipid-transfer proteins (nsLTPs), previously thought to be involved in lipid shuttling between organelles, have been implicated in plant defense[1–5], although this role does not necessarily exclude their having other functions. Plant nsLTPs are ubiquitous and form a single family of similar 90–95 amino acid polypeptides that have been identified (at the protein and/or DNA levels) in a variety of tissues from mono- and dicotyledonous species[6].

A name in search of biological meaning

Lipid-related properties of plant nsLTPs have been comprehensively reviewed by Yamada[6] and by Arondel and Kader[7]. In summary, these proteins can facilitate the transfer of a broad range of lipids from donor to acceptor membranes *in vitro*. Their proposed biological role is the cytoplasmic transfer of lipids from the endoplasmic reticulum to organelle membranes, such as those of mitochondria, which cannot synthesize phosphatidylethanolamine and phosphatidylinositol, or those of chloroplasts, which do not make phosphatidylcholine[6]. However, the cytoplasmic role originally proposed for nsLTPs seems unlikely because it is now known that they are synthesized as precursors with typical signal peptides[4,8–10] and secreted in certain cell cultures[11,12], and they have been found to be externally associated with the cell wall[1–4,13,14] (with the possible exception of a castor-bean nsLTP, for which a glyoxisomal location has been proposed[6]). These findings have led to speculation that these proteins might be involved in the secretion or deposition of extracellular lipophilic materials, such as cutin or wax[12,14,15], although additional studies are required to establish this.

From lipid transfer to inhibition of pathogens

The antibiotic properties of plant nsLTPs were discovered in a general approach to identify novel defense proteins and genes[16] that involved, as a first step, a blind screening of proteins purified from crude cell-wall preparations from different plant species[1–4]. Four proteins (LTP2–LTP5) that were isolated from barley leaves and shown to be active against the bacterial pathogen *Clavibacter michiganensis* subsp. *sepedonicus* (EC_{50} values = $1–3 \times 10^{-7}$ M) are similar in sequence to previously described nsLTPs[1,2]. These proteins are also active against other bacterial and fungal pathogens. Furthermore, all nsLTPs tested from other plant species, including those from maize, spinach, *Arabidopsis*, radish and broccoli, have antibiotic activity[1–3,5,14].

The relative activities of the different nsLTPs vary between pathogens, indicating that there is some degree of specificity[2]. Different strains of a given pathogen can have different susceptibilities to nsLTPs[16]. While nsLTPs are much more active than are thionins (another group of plant-defense proteins) against the bacterial pathogen *C. michiganensis* subsp. *sepedonicus*, the opposite is true for the fungus *Fusarium solani*. This suggests that nsLTPs might complement thionins and other defense proteins to form a general barrier against pathogens[1,2,16]. Furthermore, nsLTPs act additively with thionins against *C. michiganensis* subsp. *sepedonicus* and synergistically against *F. solani*[1,2]. Whether the mechanism of microbial inhibition is related to the lipid-transfer properties of these proteins is not yet known. Although $CaCl_2$ has been shown to antagonize the activity of nsLTPs[5], chelation of Ca^{2+} alone does not explain their inhibitory properties, as the relative activities of individual nsLTPs would then depend exclusively on their affinities for the cation (and would be independent of the pathogen), whereas the activities actually vary between pathogens.

A defense-protein shield

Early studies of plant nsLTPs suggested that there were only one or two variants per species and that their distribution was restricted to certain tissues. However, more recent findings[1–4,17] indicate that nsLTPs are encoded by widely divergent multigene families and are widely distributed in the plant, especially over exposed surfaces and in vascular tissues. For example, in barley, at least six genes (on chromo-

somes 3H, 5H and 7H) have been identified[4]. Expression of three of these genes (*Ltp2*, *Ltp3* and *Ltp4*) is detected clearly in stem, shoot apex, leaves, different parts of the spike and in the kernel (not in endosperm), while lower levels occur in roots[4]. By contrast, *Ltp1* mRNA has only been detected in the aleurone cell layer[11]. As illustrated in Fig.1, LTP2–LTP5 of barley are localized in the outer, epidermal cell layer of the exposed surfaces of the plant, as well as in the embryo and in vascular tissues, using a single polyclonal antibody that recognizes all these proteins[4]. A substantial fraction of these proteins can be extracted simply by dipping an intact leaf into a high-salt buffer[4]. Furthermore, the expression and distribution of LTP1 of *Arabidopsis*[13,15], one of several nsLTPs that are known to exist in that species[3,13], resemble those of LTP4 in barley. More recently, an nsLTP has been found with a similar distribution in the surface wax and phloem of broccoli[14].

The average concentrations of nsLTPs in barley tissues have been estimated as $>1 \times 10^{-5}$ mol kg^{-1} fresh weight, which means that the concentration at the tissue surface must be $>1 \times 10^{-4}$ mol kg^{-1} (Ref. 4). These concentrations are much higher than those required to inhibit many pathogens *in vitro*. Thus, nsLTPs appear to provide the plant with a defensive-protein shield.

Genetic responses to pathogens

As with other types of defense proteins, the expression of *Ltp* genes responds to infection by pathogens in a complex manner. The way in which the expression of three barley *Ltp* genes responds to abiotic stimuli and to different pathogens has been compared with that of the barley genes encoding thionins (*Th*) and the pathogenesis-related protein PRHv-1 (*PrHv-1*)[4,16] (Table 1). Expression of *Ltp* genes can be

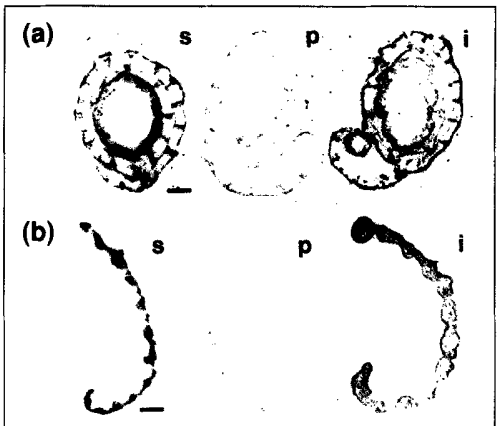

Fig. 1. Distribution of nonspecific lipid-transfer proteins as a defense-protein shield in barley[4]. The figure shows tissue-printing detection in sections of the barley plant using **(I)** an anti-barley-LTP2/LTP4 polyclonal antibody that recognizes LTP2–LTP5 (Ref. 4); control sections were stained with **(s)** Amido Black or **(p)** treated with pre-immune serum. **(a)** Shows a transverse section of leaves and stem in the upper part of a 90 day old plant, with the epidermal layer and vascular tissues being differentially immunostained. Scale bar = 0.4 mm. **(b)** Shows a cross-section of a leaf from a 12 day old plant, with the epidermal layer being preferentially immunostained. Scale bar = 0.5 mm.

Table 1. Level of expression of barley *Ltp* genes induced by abiotic stimuli and bacterial and fungal pathogens[4,16]

			Level of mRNA compared with normal (fold)[b]				
Stimuli	**Barley cultivar**	**Compatibility[a]**	***Ltp2***	***Ltp3***	***Ltp4***	***Th***	***PrHv-1***
Abiotic							
Salinity	Bomi		2	–	2	–	–
Cold	Bomi		–	–	–	–	–
Drought	Bomi		–	–	–	4	–
Wounding	Bomi		–	–	–	–	–
Methyl-jasmonate	Bomi		0.05	0.06	0.10	20	–
Abscisic acid	Bomi		5	2	3	3	7
Ethylene	Bomi		–	–	–	–	–
Ethephon	Bomi		–	–	–	–	3
Salicylate	Bomi		–	–	–	–	7
Isonicotinic acid	Bomi		–	–	–	2	3
Pathogen							
Erysiphe graminis							
CC142	Pallas	I	3	3	9	3	6
CC143	Pallas	C	3	3	8	3	6
Rhynchosporium secalis							
US238.1	Atlas46	I	3	–	10	4	10
US238.1	Atlas	C	–	–	–	4	–
US238.1	Turk	I	4	–	16	–	20
US238.1	Hannchen	C	–	–	–	–	–
Pseudomonas syringae							
153	Bomi	I	–	–	–	–	–
DC3000	Bomi	C	–	–	–	5	4

[a]C, compatible; I, incompatible.
[b]Expression of thionin genes (*Th*) was investigated with a probe that recognizes all known barley-leaf thionins[4] and that of *PrHv-1*, with a probe from Hahn *et al.*[19] Filters for the *E. graminis* interaction were from Boyd *et al.*[18]

Questions

- What function(s), other than their defensive role, do nonspecific lipid-transfer proteins (nsLTPs) have in plants?
- What mechanism(s) are responsible for the toxicity of nsLTPs to pathogens?
- What mechanisms are responsible for possible microbial resistance to nsLTPs?
- Are nsLTPs essential for plants?
- Are nsLTPs a lead for drug development?

induced well above basal levels, or switched off, independently from each other and from the genes used for comparison. Different gene combinations are affected by the various abiotic treatments and by pathogens (Table 1), indicating that multiple elicitation pathways are stimulated.

Infection by the fungus *Erysiphe graminis* affects the expression of all the genes (*Ltp*, *Th* and *PrHv-1*; Table 1). The observed increases in mRNA levels are essentially the same in the compatible (successful) and incompatible (unsuccessful) interactions[4,16], and occur within the first few hours after inoculation, before the infection by the avirulent strain (incompatible interaction) is stopped[18]. In this case, incompatibility (resistance) must depend either on products of other defense genes or on a greater susceptibility of the avirulent strain to one or more of the gene products under investigation (such as nsLTPs, thionins and PRHv-1).

On infection of barley with the fungal pathogen *Rhynchosporium secalis*, the incompatible reaction (and not the compatible one) leads to an increase above the basal level of *Ltp4* mRNA and, to a lesser extent, of *Ltp2* mRNA, concomitantly with the increase in *PrHv-1* mRNA (Table 1). These observations were made on the same RNA preparations used previously by Hahn *et al.*[19] to investigate the induction of *PrHv-1* in response to the same fungus. The barley Atlas46 cultivar has a gene (*Rh3*, transferred from the Turk cultivar) that confers resistance to *R. secalis*, while the nearly isogenic Atlas cultivar lacks this gene. This means that the *Ltp2*, *Ltp4* and *PrHv-1* genes are under the control of the *Rh3* gene, and that the proteins encoded might be responsible for the resistance of the Atlas46 cultivar to the fungus (Table 1). For the incompatible interaction of *Pseudomonas syringae* 153 with the Bomi cultivar, in which no hypersensitive reaction is detected, resistance to the bacterial pathogen could be due to the presence of high basal levels of the defense proteins. Furthermore, successful infection (compatible interaction) by *P. syringae* pv. *japonica* switches off the expression of the three *Ltp* genes, and successful infection by *Xanthomonas campestris* pv. *translucens* of the barley Bomi cultivar increases the level of only the *Ltp4* mRNA (A. Molina and F. García-Olmedo, unpublished).

In summary, the expression of the *Ltp* genes (especially that of *Ltp4*) responds to pathogens in a way that is consistent with their products having a defensive role. However, the response might be irrelevant if the proteins that are elicited are not inhibitory (individually or in combination) to the pathogen that elicits them. Furthermore, a successful infection might result not only from the plant failing to recognize a particular pathogenic strain and therefore failing to activate the defense system (that is, a compatible interaction), but also from the ability of a specific strain of pathogen to resist the action of inhibitor(s) to which other pathogenic strains are susceptible (that is, susceptible strains would be incompatible).

Conclusion

Plant nsLTPs have the necessary inhibitory properties, the appropriate distribution and concentration in the plant and the correct patterns of gene expression under pathogen attack to be considered active defense proteins. This role does not exclude the possibility that some or all of the members of this protein family that are present in a given plant might have other functions in response to various stresses[20–22], during development[12] or during the course of normal metabolism[6,7].

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